In the Office Action dated September 7, 2000, claims 1-11, 13-17, 25 and 26 were rejected under 35 U.S.C. §112(1). Claims 2-11, 13-17, 25 and 26 were rejected under 35 U.S.C. §112, second paragraph. Claims 11 and 13-16 were rejected under 35 U.S.C. §102(b) as being allegedly anticipated by Shimizu et al. In the present Amendment, claims 1, 2, 4, 7-11, 14, 15, 25 and 26 have been amended. Support can be found in the specification for any amendments to existing claims. Thus, no new matter is added.

Claim to Right of Priority

The Official Action alleged that Applicants have not complied with 35 U.S.C. §119(e) sufficient to obtain benefit of an earlier filing date. Applicants have amended the specification as required to include a specific reference to the prior application upon which priority is based. Withdrawal of rejection is respectfully requested.

Rejections under 35 U.S.C. §112(1)

Claims 1-11, 13-17, 25 and 26 were rejected under 35 U.S.C. §112, first paragraph. In particular, the Office Action alleged that it is not clear from the specification that the Applicant was in possession of the invention as claimed.

The Applicants traverse this rejection as it applies to claims 1-11, 13-17, 25 and 26 as presently amended. The requirement under Section 112, first paragraph, that the specification contain "a written description of the invention" raises a factual issue: "Does the specification convey clearly to those skilled in the art, to whom it is addressed, in any way, the information that [applicants] invented that specific [claimed invention]?" Vas-Cath, 19 USPQ2d at 1115 (citing In re Rushchig, 154 USPQ 118, 123 (CCPA 1967)) (emphasis added). Under the uniform standard for determining compliance with that requirement, the U. S. Court of Appeals for the Federal Circuit has stated unequivocally, the applicant does not have to describe exactly the subject matter claimed. The description must be sufficient merely to allow a person of ordinary skill in the art to recognize that the applicant invented what is claimed. Vas-Cath, 19 USPQ2d at 1116 (citing In re Gosteli, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989)).

The PTO Board of Patent Appeals and Interferences has also applied the uniform standard. The Board, in In re Hold, 19 USPQ2d 1211 (Bd. Pat. App. & Inter. 1991), reversed a rejection under 35 U.S.C. §112(1) wherein the examiner contended that the specification failed to provide a written description adequate to support the claims. The Board stated, "It is well established that the invention claimed need not be described *ipsis verbis* in order to satisfy the disclosure requirement of Section 112." Id. at 1213.

The disclosure of the present application is clearly sufficient to convey to a person of ordinary skill in the art that the applicants invented the claimed subject matter. Section 112, first paragraph, requires nothing more. In particular, the disclosure of SEQ ID NO:1 combined with hybridization protocols (page 23, lines 16-33 and page 14, lines 1-31), chromosomal walking procedures (page 23, line 34 through page 24, line 18), screening and identification of homologs from numerous *M. grisea* stains (page 30, lines 5-15), and procedures to examine function as described in Examples 3 and 4 (pages 30-31) provide ample evidence that the applicants invented the entire subject matter of claim 1. Withdrawal of rejection is respectfully requested.

Claims 1-11, 13-17, 25 and 26 were also rejected under 35 U.S.C. §112, first paragraph because the specification was allegedly enabling only for claims limited to an isolated nucleic acid molecule encoding SEQ ID NO:4. The Office Action appeared to base the rejection on the premise that "Applicant has provided no guidance with respect to what hybridization/wash conditions or what PCR reaction conditions would allow specific isolation of additional functionally related genes.

The Applicants respectfully disagree with this allegation. The specification is enabling for one of ordinary skill in the art to make and use the invention as claimed in claim 1, as amended. Specifically, one of ordinary skill in the art would know how to isolate a segment approximately 1 kb in size from chromosome 1 of *Magnaporthe grisea*. Further, the ordinary artisan would know how to use detailed examples in the specification to determine if this 1kb segment confers rice cultivar CO39-specific avirulence to fungal plant pathogens. With respect to claim 2, detailed disclosure of hybridization and wash conditions and corresponding formulas are provided in the

Thus, one of ordinary skill in the art would be easily able to make and use the invention as recited in amended claim 1 and claims dependent upon claim 1 without undue experimentation. Withdrawal of rejection is respectfully requested.

Claims 7, 8 and 15 were rejected for allegedly reading on mammalian cells *in vivo*. Claims 7 and 14 have been amended to recite, "A non-mammalian cell..." Thus, amended claims 7, 8, and 15 do not read on mammalian cells *in vivo*. Further, the process of transforming insect cells is well known for use with DNA of any kind. As set forth in MPEP §2164.01, "A patent need not teach, and preferably omits, what is well known in the art." (citing In re Buchner, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991). One of ordinary skill in the art would be able to transform an insect cell with the DNA of the present invention. Clontech, for example, sells an entire baculovirus expression system kit, a rapid titer kit, associated viral DNA, and even insect cell media. Copies of relevant pages from the 2000 Clontech catalog are enclosed herewith. Applicants assert that one of ordinary skill in the art would be able to transform the DNA of the present invention into an insect cell without undue experimentation. Thus, claims 7, 8, and 15 are enabled by the specification combined with the state of the art. Withdrawal of rejection is respectfully requested.

Rejections under 35 U.S.C. §112(2)

Claims 2-11, 13-17, 25 and 26 were rejected under 35 U.S.C. §112, second paragraph as being allegedly indefinite.

Claim 2 was rejected as being indefinite for reciting the name, "AVR1-CO39." In particular, the Office Action alleged that AVR1-CO39 does not clearly identify the claimed gene and does not set forth the metes and bounds of the claimed invention.

Claim 2, as amended, does not recite, "AVR1-CO39." Thus, this rejection is rendered moot. Support for this amendment may be found in the specification from page 10, line 20, through page 11, line 22, and page 12, lines 5-10. Withdrawal of rejection is respectfully requested.

Claim 4 was rejected as indefinite for reciting the term, "having the features of a

Claim 6 was rejected as being allegedly indefinite for reciting a vector, when according to the Office Action, a vector is a circular piece of DNA and "is it not known how a recombinant DNA can comprise both a vector and another nucleic acid molecule and how the vector and nucleic acid molecule are attached to one another."

The Applicants respectfully traverse this rejection as it applies to claim 6. The procedure for operably linking DNA sequences in vectors is routine in the art and well understood. As is also understood in the art, a vector need not necessarily be circular in form. As stated in Singer & Berg (Genes & Genome, University Science Books, Mill Valley, CA, p262, copy enclosed), a vector is "a DNA molecule that can replicate in an appropriate host cell," and "any extrachromosomal small genome (like those of plasmids, phage and viruses) is, in principle, a potential vector." Not all viruses or phage are circular in form. However, even for vectors that are circular, such as plasmids, the Applicants assert that the procedure for operably linking recombinant DNA to vectors is routine to one of ordinary skill in the art. For example, Sambrook, Molecular Cloning, A Laboratory Manual, provides an entire chapter on cloning DNA into plasmid vectors (see Volume 1, chapter 1, copy of relevant selections enclosed). Thus, claim 6 is sufficiently definite in view of the state of the art and description of "operably linked" on page 9, lines 5-13. Withdrawal of rejection is respectfully requested.

Claim 8 was rejected for reciting "cell" at line 1 but reciting "cells" at lines 2-3. Applicants have amended claim 8 to recite, "The cell of claim 7, wherein said cell is either bacterial, fungal, insect, or plant." Withdrawal of rejection is respectfully requested.

Claims 9 and 10 were rejected for allegedly lacking antecedent basis for the term, "transformed cell." Applicants point out that claim 7 recites "A cell transformed..." thus providing adequate antecedent basis for claims 9 and 10. Nevertheless, claims 9 and 10 have been amended to no longer recite, "a transformed cell." Withdrawal of rejection is respectfully requested.

Claim 11 was rejected for reciting the term, "substantially the same." Claim 11

Claim 15 was rejected for reciting "cell" at line 1 but reciting "cells" at lines 2-3. Applicants have amended claim 15 to recite, "The cell of claim 14, wherein said cell is either bacterial, yeast, insect or plant." Withdrawal of rejection is respectfully requested.

Claim 25 was rejected as indefinite for reciting the phrase "effective to confer." Claim 25 has been amended as required by the Examiner to recite, "which confers." Withdrawal of rejection is respectfully requested.

Claim 26 was rejected for being dependent on cancelled claim 24 and for reciting the term, "functional equivalent." Claim 26 has been amended to be dependent on claim 25 and to recite, "or an allelic variant thereof." Claim 26 has also been amended to recite, "the amino acid sequence of SEQ ID NO:4." The amino acid sequence of SEQ ID NO:4 is essentially the same subject matter as "expresses ORF3 of SEQ ID NO:1." Support can be found in the specification on page 10, lines 25-29, and in disclosure of SEQ ID NO:1 and SEQ ID NO:4. Withdrawal of rejection is respectfully requested.

Rejections under 35 U.S.C. §102(b)

Claims 11 and 13-16 were rejected under 35 U.S.C. §102(b) as being allegedly anticipated by Shimizu et al. The Office Action alleged that Shimizu et al. discloses an isolated nucleic acid molecule which has part of SEQ ID NO:1, which hybridizes with part of SEQ ID NO:1, or which encodes part of any of SEQ ID NO:2-8. Further, the Office Action alleged that Shimizu et al. discloses a recombinant DNA comprising the nucleic acid molecule and an *E. coli* host cell transformed with the nucleic acid molecule.

Applicants traverse this rejection. A claim is anticipated by a reference only if each and every element of the claim is found, either expressly or inherently, in that reference. *MPEP 2131*. Moreover, the identical invention must be shown in as complete detail as is contained in the claim. *Id.* The Examiner did not point out which sequence or sequences in Shimizu et al. formed the basis of this rejection. Therefore, Applicants assume that this rejection was based on recitation of the language "or part

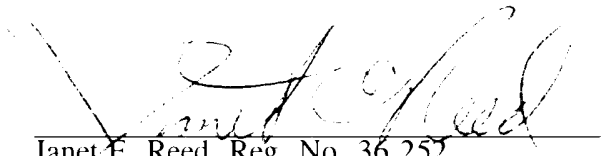
Claim 11 as amended no longer recites the term, "or part of." Abiding by these standards, the Shimizu et al. reference clearly does not anticipate the invention as claimed in claims 11 as amended and therefore claims 13-16, which are dependent on claim 11. Withdrawal of rejection is respectfully requested.

Summary

In view of the foregoing amendments and remarks, the Applicants submit that this application is in condition for allowance and respectfully request early and favorable notification to that effect. If it would expedite prosecution of this application, the Examiner is invited to confer with Applicants' undersigned attorney.

The Applicants reserve the right to prosecute, in one or more divisional or continuation applications, the claims as originally filed and all other claims supported by the specification.

Respectfully Submitted,



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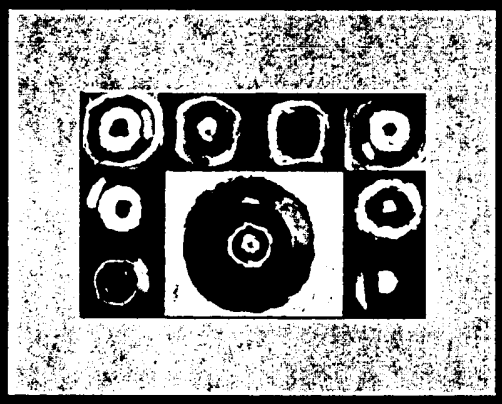
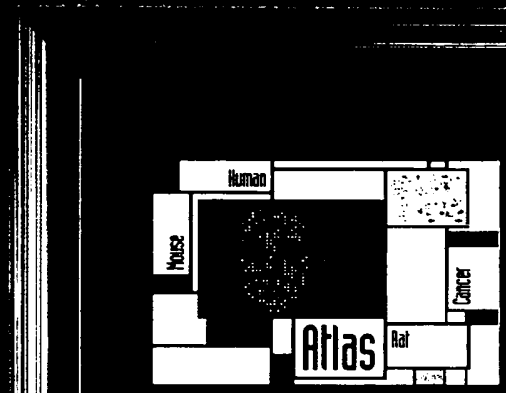
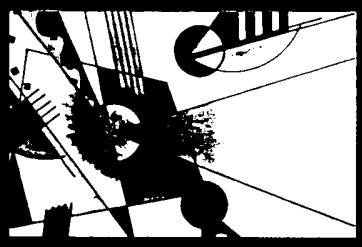
CLONTECH





INNOVATIVE
TOOLS TO
ACCELERATE
DISCOVERY



BacPAK™ Baculovirus Expression System

- *Expresses proteins at high levels—1 to 500 mg of protein per liter of culture*
- *Retains the biological activity of expressed proteins*
- *High recombinant efficiency*

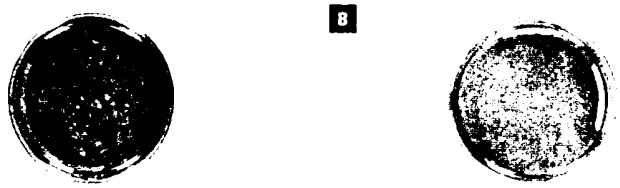


Figure 1. Relative efficiency of producing recombinant viruses using wild-type or BacPAK6 viral DNAs. Sf21 cells were cotransfected with pBacPAK8-GUS (a transfer vector containing the *E. coli* β-glucuronidase [GUS] gene) and either circular wild-type AcMNPV DNA (Panel A) or *Bsu*36 I-digested BacPAK6 DNA (Panel B). Viruses produced after 48–72 hr were harvested and plated to obtain individual plaques. In the presence of X-Gluc and neutral red, recombinant plaques turn blue, nonrecombinant plaques appear white, and uninfected cells stain red.

The BacPAK™ Baculovirus Expression System expresses recombinant proteins at extremely high levels in insect host cells (1, 2). BacPAK offers three major advantages:

- **High yield of recombinant protein.** The insect host cells produce large amounts of your target protein (Table I).
- **Greater similarity to naturally occurring proteins.** The expressed recombinant protein is usually similar in structure, biological activity, and immunological reactivity to the naturally occurring protein because insect host cells provide posttranslational processing similar to that of mammalian cells.
- **High recombination efficiency.** More than 90% of the viruses produced by the transfected cells carry the target protein (Table II). The specially designed BacPAK6 viral DNA forces recombination between the virus and transfer vector, resulting in high recombination efficiency.

Table I: Comparison of Protein Production in Various Expression Systems

Expression system	Insect cells	*E. coli*	Yeast cells	Mammalian cells
Proteolytic cleavage	+	+/–	+/–	+
Glycosylation	+	–	+	+
Secretion	+	+/–	+	+
Folding	+	+/–	+/–	+
Phosphorylation	+	–	+	+
Acylation	+	–	+	+
Amidation	+	–	–	+
Percent Yield	30			

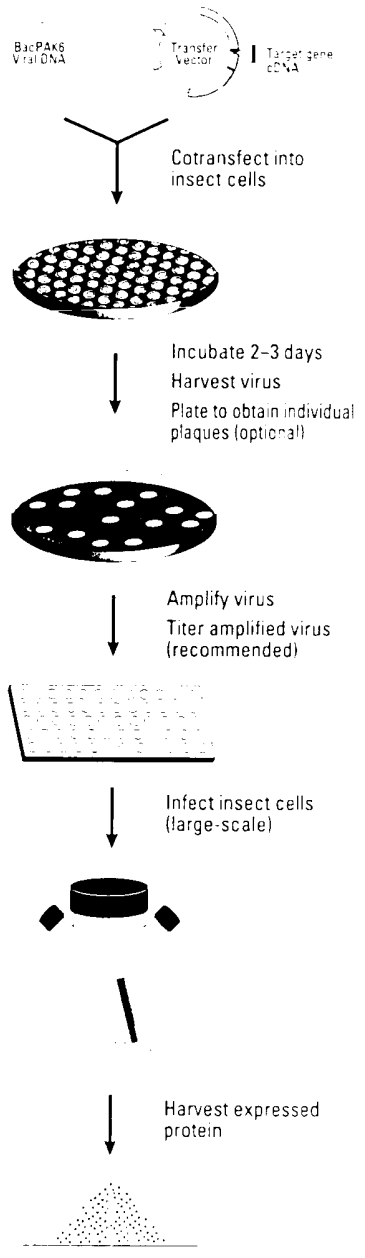


Figure 2. The BacPAK Baculovirus System

BacPAK™ Baculovirus Expression System continued

BacPAK™ method

The target gene is inserted into a transfer vector, which is cotransfected into insect host cells with the linearized BacPAK6 viral DNA. The BacPAK6 DNA is missing an essential portion of the baculovirus genome. When the DNA recombines with the vector, the essential element is restored and the target gene is transferred to the baculovirus genome. Following recombination, a few viral plaques are picked and purified, and the recombinant phenotype is verified. The newly isolated recombinant virus can then be amplified and used to infect insect cell cultures to produce large amounts of the desired protein.

The BacPAK System includes the transfer vectors, viral DNA, and insect host cells needed for production of recombinant proteins and Bacfectin™ Transfection Reagent for highly efficient transfections. A User Manual, sequencing/PCR primers, and control viruses are also included with the kit. To rapidly determine baculovirus titers, CLONTECH offers the BacPAK Rapid Titer Kit (page 166).

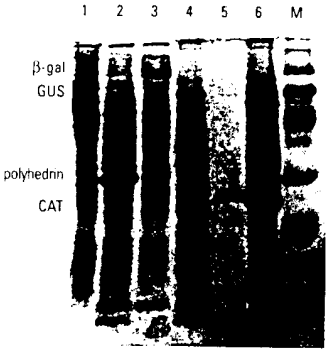


Figure 3. Protein production from recombinant viruses generated using the BacPAK™ Baculovirus Expression System. Recombinant viruses were obtained by cotransfection of transfer vectors with BacPAK6 Viral DNA (*Bsu*36 I digest), followed by amplification in Sf21 cells. Lane 1: uninfected Sf21 cells. Lane 2: Sf21 cells infected with wild-type AcMNPV virus. Lane 3: Sf21 cells infected with nonrecombinant BacPAK6 virus. Lane 4: Sf21 cells infected with BacPAK8-GUS recombinant virus. Lane 5: purified CAT protein. Lane 6: Sf21 cells infected with BacPAK9-CAT recombinant virus. Lane M: molecular weight markers. The SDS PAGE analysis was performed 48 hr after infection of the Sf21 cultures.

Product	Size	Cat. #	Price
BacPAK Baculovirus Expression System	each	K1601-1	$545.00

STORAGE CONDITIONS

−180°C for IPLB-Sf21 Cells
−70°C for BacPAK6 & AcMNPV Virus Stocks
4°C for BacPAK6 Viral DNA & Bacfectin
−20°C for all other components

KIT COMPONENTS

pBacPAK8 Transfer Vector
pBacPAK9 Transfer Vector
BacPAK6 Viral DNA *(Bsu36 I digest)*
Bacfectin™ Transfection Reagent
IPLB-Sf21 *Spodoptera frugiperda* Cells
BacPAK6 Virus Stock *(positive control)*
AcMNPV Wild-Type Virus *(negative control)*
Bac1 Sequencing/PCR Primer
Bac2 Sequencing/PCR Primer
pBacPAK8-GUS Positive Control Transfer Vector
User Manual *(PT1260-1)* & Protocol-at-a-Glance *(PT1260-2)*

REFERENCES

1. Kitts, P. A. & Possee, R. D. (1993) *BioTechniques* **14**(5): 810–817.
2. Kitts, P. A., *et al.* (1990) *Nucleic Acids Res.* **18**: 5667–5672.

Table II: Comparison of Production of Recombinant Virus

Viral DNA	Transfer vector with insert	Plaques examined	Recombinant plaques	Percent recombinant
BacPAK6 DNA digest	pBacPAK9-CAT	75	75	100%
	pBacPAK1-sgp[a]	27	26	96%
	pAc*Eco*R I-I-actin[b]	10	10	100%
	pBacPAK8-GUS	93	85	91%
Linear viral DNA	pBacPAK8-GUS	275	80	29%
Wild-type DNA (circular)	pBacPAK8-GUS	322	9	2.8%

[a] Data from I. Kuhn, Berlex Biosciences
[b] Data from K. Storm, UC Berkeley

BacPAK™ Rapid Titer Kit

- *Saves time by shortening baculovirus expression experiments up to six days*
- *Eliminates troublesome plaque assays*
- *Compatible with all AcMNPV-based baculovirus expression systems*

Product	Size	Cat. #	Price
BacPAK Baculovirus Rapid Titer Kit	5 assays	K1599-1	$260.00

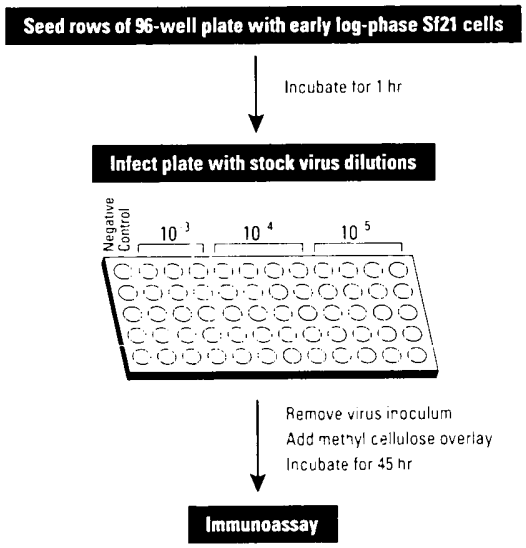


Figure 1. The BacPAK Baculovirus Rapid Titer Kit procedure

KIT COMPONENTS

Mouse gp64 Antibody
Goat Anti-Mouse Antibody/HRP Conjugate
Blue Peroxidase Substrate
Normal Goat Serum
Methyl Cellulose Overlay
Resealable Plastic Bags
Control Baculovirus
User Manual *(PT3153-1)*

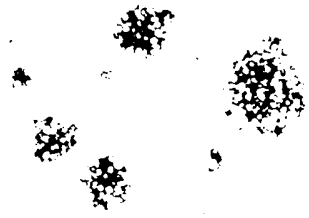


Figure 2. Detection of foci of infection in a lawn of Sf21 cells with the BacPAK™ Baculovirus Rapid Titer Kit. Cells were immunostained using HRP as described in the User Manual.

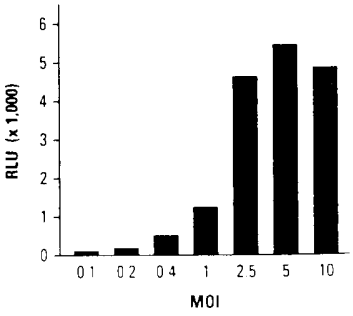


Figure 3. Correct titer can be critical for optimal protein expression. Luciferase expression was graphed as a function of multiplicity of infection (MOI). Sf21 cells were infected at different MOIs with a recombinant baculovirus expressing luciferase (2). 21 hr post-infection, the infected cells were lysed and assayed for luciferase activity. RLU = relative light units.

The BacPAK™ Baculovirus Rapid Titer Kit provides the quickest method for determining titers of baculovirus stocks, typically the most time-consuming part of baculovirus expression protocols. The kit uses a standard immunological assay to accurately determine baculovirus titers in 48 hours, whereas other methods, such as plaque and end-point dilution assays, take 4–8 days.

In baculovirus expression systems, infected cells express viral antigens long before plaques are formed. The BacPAK Rapid Titer assay allows titer determination after a much shorter incubation period (1). Furthermore, the titers obtained are comparable to those obtained with other methods. This kit is suitable for use with any virus stock with a titer of more than 10^4 pfu/ml and is compatible with all AcMNPV-based baculovirus expression systems.

The Rapid Titer immunoassay uses a primary monoclonal antibody raised against an AcMNPV envelope glycoprotein (gp64) to accurately identify virally infected cells. A secondary HRP-conjugated antibody enables you to visualize infected cells by light microscopy and determine viral titer.

The BacPAK Rapid Titer Kit includes all the necessary reagents, except organic solvents, and control baculovirus to perform five titration assays.

[illegible] Baculovirus
Room temperature for all other components

REFERENCES

BacPAK6 Viral DNA

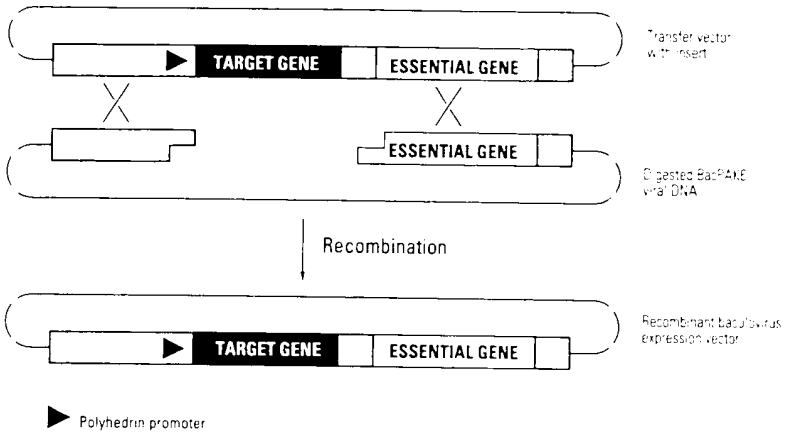
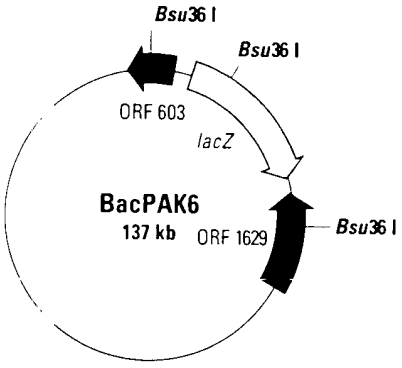


Figure 1. Recombination of transfer vector and BacPAK6 Viral DNA. Sf21 cells are cotransfected with an appropriate transfer vector and BacPAK6 DNA (*Bsu*36 I digest). The target gene, which has been cloned into the transfer vector, is transferred to the baculovirus expression vector by forced recombination.

Product	Size	Cat. #	Price
BacPAK6 DNA (*Bsu*36 I digest)	5 transfxns	6144-1	$310.00



BacPAK6 Viral DNA facilitates the production of recombinant viral expression vectors from AcMNPV transfer vectors. The use of *Bsu*36 I-digested BacPAK6 DNA yields recombinant viruses at a frequency of 90% or higher (1).

When BacPAK6 is digested with *Bsu*36 I, a small fragment of an essential gene is released. The remaining large fragment is not capable of producing viral plaques; however, recombination with a compatible transfer vector restores the integrity of the gene and allows production of viable recombinant virus. Because of this lethal deletion, BacPAK6 DNA digest provides a selection for recombinant viruses.

BacPAK6 also carries the *lacZ* gene, which is driven by the polyhedrin promoter, so that plaques of the parental virus can be detected using X-α-Gal (page 240).

BacPAK6 DNA (*Bsu*36 I digest) is provided with Bacfectin™ transfection reagent for efficient cotransfections.

STORAGE CONDITIONS: 4 C

REFERENCE

1. Kitts, P. A. & Possee, R. D. (1993) *BioTechniques* 14(5) 810–817.

BacPAK™ Insect Cell Media

Complete and Basic Media formulated especially for use with baculovirus systems

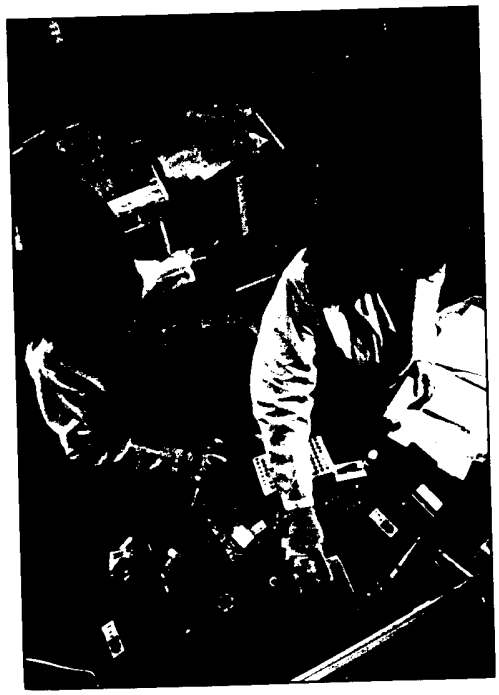
Product	Size	Cat. #	Price
BacPAK Complete Medium	1 L	8090-1	$165.00
BacPAK Grace's Basic Medium	500 ml	8091-1	$50.00

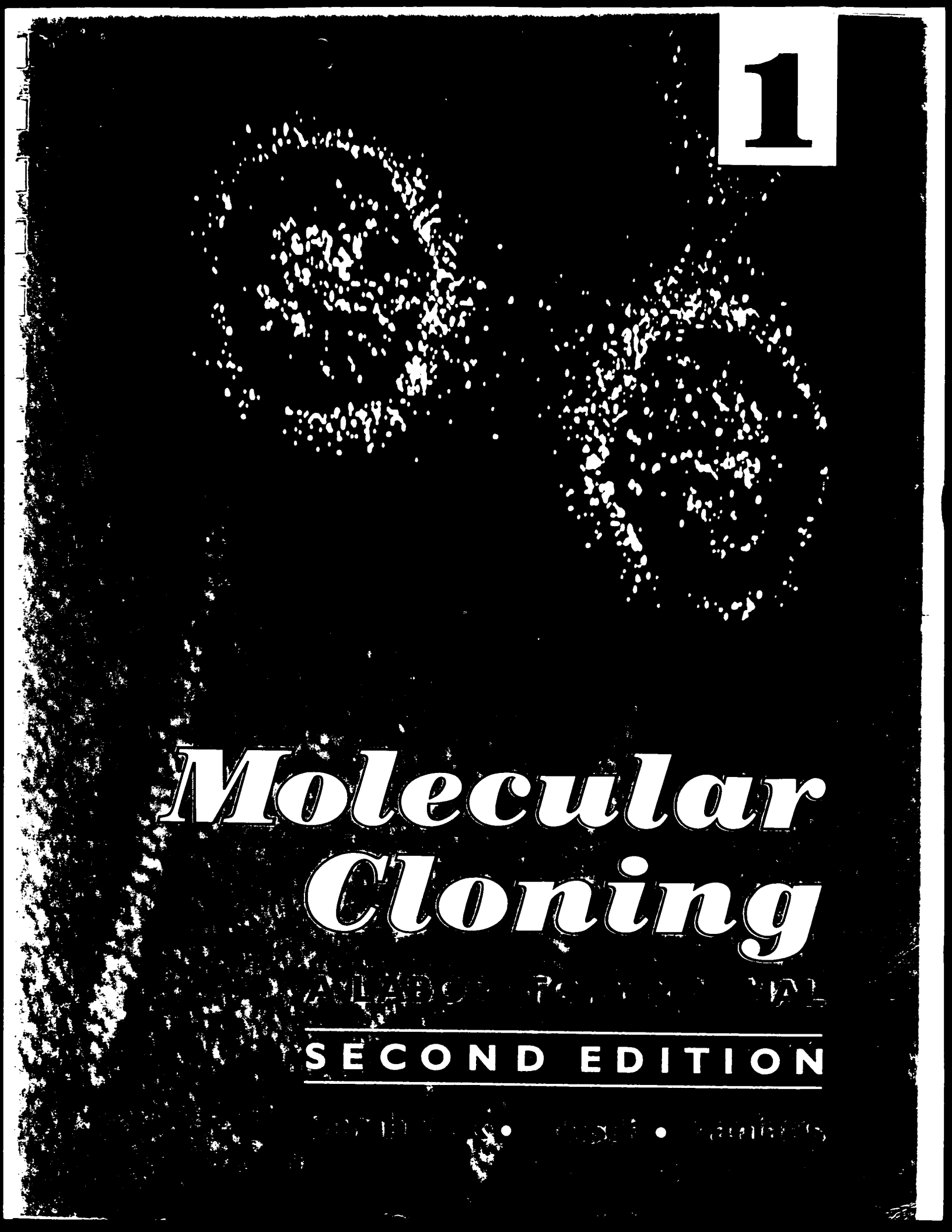
CLONTECH offers two media formulated for the special requirements of baculovirus expression systems: BacPAK™ Complete Medium and BacPAK™ Grace's Basic Medium.

BacPAK™ Complete Medium is fully supplemented and provides ideal growth conditions for the *Spodoptera frugiperda* cells (Sf21 or Sf9) used with the BacPAK Baculovirus Expression System (pages 164–165) and other baculovirus-based systems. This medium is based on TNM-FH and contains 10% fetal bovine serum and gentamicin. It is ready to be used without any supplementation.

BacPAK™ Grace's Basic Medium is a base medium without any proteinaceous supplements. Because the presence of protein can inhibit transfection, this medium maximizes the efficiency of transfection. BacPAK Grace's Basic Medium can easily be supplemented to make TNM-FH medium.

STORAGE CONDITIONS: 4°C




1
Molecular
Cloning
A LABO
SECOND EDITION

Ligation of Cohesive Termini

1. Digest the plasmid and foreign DNAs with the appropriate restriction enzymes. If necessary, isolate the desired fragments by gel electrophoresis and/or treat the plasmid DNA with phosphatase. Purify the DNAs by extraction with phenol:chloroform and ethanol precipitation. Redissolve the DNAs in TE (pH 7.6) at a concentration of 100 μg/ml.

2. Set up the ligation mixtures as follows:

   a. Transfer 0.1 μg of the vector DNA to a sterile microfuge tube. Add an equimolar amount of foreign DNA.

   b. Add H_2O to 7.5 μl, and warm the solution to 45°C for 5 minutes to melt any cohesive termini that have reannealed. Chill the mixture to 0°C.

   c. Add:

10× bacteriophage T4 DNA ligase buffer	1 μl
bacteriophage T4 DNA ligase	0.1 Weiss unit
5 mM ATP	1 μl

   Incubate the reactions for 1–4 hours at 16°C.

10× Bacteriophage T4 DNA ligase buffer

200 mM Tris·Cl (pH 7.6)
50 mM $MgCl_2$
50 mM dithiothreitol
500 μg/ml bovine serum albumin (Fraction V; Sigma) (optional)

This buffer should be stored in small aliquots at −20°C.

Set up two additional control reactions that contain (1) the plasmid vector alone and (2) the fragment of foreign DNA.

If insufficient foreign DNA is available, use 50–100 ng of the plasmid DNA per ligation and add as much foreign DNA as possible without increasing the volume of the ligation reaction to greater than 10 μl.

At least three different assays are used to measure the activity of bacteriophage T4 DNA ligase. Most manufacturers (apart from New England Biolabs) now calibrate the enzyme in Weiss units (Weiss et al. 1968). One Weiss unit is the amount of enzyme that catalyzes the exchange of 1 nmole of ^{32}P from pyrophosphate into [γ,β-^{32}P]ATP in 20 minutes at 37°C. One Weiss unit corresponds to 0.2 unit as determined by the exonuclease resistance assay (Modrich and Lehman 1970) and to 60 cohesive-end units as defined by New England Biolabs. 0.015 Weiss unit of bacteriophage T4 DNA ligase therefore will ligate 50% of the *Hin*dIII fragments of bacteriophage λ (5 μg) in 30 minutes at 16°C. Throughout this manual, bacteriophage T4 DNA ligase is given in Weiss units.

Bacteriophage T4 DNA ligase is supplied in concentrated solution (1–5 units/μl). This should be diluted and stored at a concentration of 100 units/ml in 20 mM Tris·Cl (pH 7.6), 60 mM KCl, 5 mM dithiothreitol, 500 μg/ml bovine serum albumin, 50% glycerol. Bacteriophage T4 DNA ligase is stable at this [illegible]

3. Use 1–2 μl of each of the ligation reactions to transform competent *E. coli* as described on pages 1.77–1.81 or 1.82–1.84.

Ligation of Blunt-ended DNA

Bacteriophage T4 DNA ligase, unlike *E. coli* DNA ligase, can catalyze the ligation of blunt-ended fragments of DNA (Sgaramella and Khorana 1972; Sgaramella and Ehrlich 1978). Since DNA can easily be rendered blunt-ended, this is an extremely useful property because it allows any DNA molecule to be ligated to any other. However, ligation of blunt-ended termini is a comparatively inefficient reaction and requires four conditions:

- Low concentrations (0.5 mM) of ATP (Ferretti and Sgaramella 1981)
- The absence of polyamines such as spermidine
- Very high concentrations of ligase (50 Weiss units/ml)
- High concentrations of blunt-ended termini

CONDENSING AGENTS

The problem of attaining adequate concentrations of blunt-ended DNA can be avoided by including in the reaction mixture substances that increase macromolecular crowding and cause DNA molecules to condense into aggregates, for example, polyethylene glycol (Pheiffer and Zimmerman 1983; Zimmerman and Pheiffer 1983; Zimmerman and Harrison 1985) or hexamminecobalt chloride (Rusche and Howard-Flanders 1985). These substances have two effects on ligation reactions:

1. They accelerate the rate of ligation of blunt-ended DNA by 1 to 3 orders of magnitude. This increase allows ligation reactions to be formed at low enzyme and DNA concentrations.
2. They alter the distribution of ligation products. Intramolecular ligation is suppressed, and the ligation products are created exclusively by intermolecular joining events. Thus, even at concentrations of DNA that favor circularization ($j:i = 10$), all the DNA products are linear multimers.

The following information may be useful when setting up ligation reactions that contain condensing agents.

Polyethylene glycol (PEG 8000)

- Stock solutions (40%) of PEG 8000 made in deionized water may be stored frozen in small aliquots, but they should be thawed and warmed to room temperature before being added to the ligation mixture. Maximal stimulation is observed in ligation mixtures containing 15% PEG 8000. Prepare reaction mixtures at 0°C by mixing all components except the PEG 8000 and the bacteriophage T4 DNA ligase. Then add an appropriate volume of PEG 8000 (at room temperature) and mix. Add the enzyme and incubate the reaction at 20°C.
- Maximum stimulation of ligation is observed when the ligation mixture

concentration of ATP or decrease in the concentration of $MgCl_2$ greatly reduces the degree of stimulation (Pheiffer and Zimmerman 1983).

- PEG 8000 at a concentration of 15% stimulates ligation of DNA molecules carrying cohesive termini by a factor of 10–100. The major products of the reaction are tandem concatemers.
- PEG 8000 stimulates blunt-end ligation of synthetic oligomers as short as 8 nucleotides in length. In this respect, it differs from hexamminecobalt chloride.

Hexamminecobalt chloride

- Hexamminecobalt chloride is stored as a 10 mM stock solution in water at −20°C. Stimulation of ligation by hexamminecobalt chloride is highly concentration-dependent and is maximal in ligation mixtures containing the salt at a concentration of 1.0–1.5 μM. Hexamminecobalt chloride increases the efficiency of blunt-end ligation about 50-fold, whereas ligation of cohesive termini is increased only 5-fold (Rusche and Howard-Flanders 1985).
- In the presence of monovalent cations (30 mM KCl), some stimulation of blunt-end ligation still occurs, but the distribution of ligation products changes. Instead of ligation products that are derived exclusively from intermolecular reactions, circular DNA molecules now predominate.
- Unlike PEG 8000, hexamminecobalt chloride does not significantly increase the rate of ligation of synthetic oligonucleotides.

Rapid Cloning in Plasmid Vectors

The slowest step in cloning in plasmids is the electrophoretic purification of the desired restriction fragment of foreign DNA and the appropriate segment of plasmid DNA. In the protocol given below (adapted from Struhl 1985 by S. Michaelis, pers. comm.), ligation of plasmid and foreign DNAs is carried out directly in the melted slabs of agarose recovered from the gel used for purification. The method works for both blunt-end ligation and ligation of cohesive termini, although it requires a large amount of ligase and its efficiency is about an order of magnitude lower than the standard procedure.

1. Digest the foreign DNA with the appropriate restriction enzyme(s). The amount of foreign DNA digested should be sufficient to yield approximately 0.2 μg of the target fragment. The digestion should be carried out in a volume of 20 μl or less. In a separate tube, digest 0.5 μg of the vector DNA with the appropriate restriction enzyme(s) in a total reaction volume of 20 μl or less.

   If the vector DNA carries identical cohesive termini, it should be treated with phosphatase as follows: When digestion with the restriction enzyme(s) is complete, add 2.5 μl of 100 mM Tris · Cl (pH 8.3), 10 mM $ZnCl_2$. Add 0.25 unit of calf intestinal alkaline phosphatase and incubate for 30 minutes at 37°C.

2. Separate the desired fragments by electrophoresis on an agarose gel. The gel must be cast with low-melting-temperature agarose and it must be poured and run in 1× TAE electrophoresis buffer (see Chapter 6, page 6.7) containing ethidium bromide (0.5 μg/ml) rather than the conventional 0.5× TBE.

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described on pages 1.49–1.50.

3. Examine the gel by long-wavelength ultraviolet illumination. From the relative fluorescent intensities of the desired bands, estimate the amounts of DNA that they contain (see Appendix E). Using a razor blade, cut out the desired bands in the smallest possible volume of agarose (usually 40–50 μl). Place the excised slices of gel in separate, labeled microfuge tubes.

   Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

4. Heat the tubes to 70°C for 10–15 minutes to melt the agarose.

5. Combine aliquots of the melted gel slices in a fresh tube prewarmed to 37°C. The final volume of the combined aliquots should be 10 μl or less, and the molar ratio of foreign DNA:plasmid vector should be approxi-

containing the plasmid vector alone and the other containing only the fragment of foreign DNA.

6. Incubate the three tubes for 5–10 minutes at 37°C, and then add to each tube 10 μl of ice-cold 2× bacteriophage T4 DNA ligase mixture. Mix the contents of the tubes well before the agarose hardens. Incubate the reactions for 12–16 hours at 16°C.

   2× Bacteriophage T4 DNA ligase mixture is prepared as follows:

1 M Tris · Cl (pH 7.6)	1.0 μl
100 mM $MgCl_2$	1.0 μl
200 mM dithiothreitol	1.0 μl
10 mM ATP	1.0 μl
H_2O	5.5 μl
bacteriophage T4 DNA ligase	1 Weiss unit

   Mix the components in a tube stored in an ice bath.

For definition of Weiss unit, see page 1.68.

7. Towards the end of the ligation, remove from storage at −70°C three tubes containing 200 μl of frozen competent *E. coli* each (see page 1.80). As soon as the cells have thawed, place them in an ice bath. Immediately proceed to step 8.

8. Remelt the agarose in the ligation mixtures by heating them to 70°C for 10–15 minutes.

9. Immediately add 5 μl of one of the ligation mixtures to 200 μl of competent *E. coli*. Mix the contents of the tube quickly by gentle shaking. Repeat this procedure with 5 μl taken from each of the remaining ligation mixtures. Store the transformation mixtures on ice for 30 minutes.

10. Proceed with the remainder of the transformation protocol (step 13, page 1.80).

GENES & GENOMES

A CHANGING PERSPECTIVE

Molecular cloning requires that the DNA fragment to be cloned (the insert) be joined to another DNA molecule (the vector) that can replicate in an appropriate host cell (Figure II.12). The joining is accomplished *in vitro*, and the resulting recombinant DNA molecules are then introduced into the cells. The vector molecule must contain an origin of DNA replication, and the process depends on replication functions (enzymes and other proteins) provided by the host cell or encoded on the vector itself. Any extrachromosomal small genome (like those of plasmids, phage, and viruses) is, in principle, a potential vector. Each of these genomes is found in nature within a particular species and, for the most part, replicates only within its natural host or cells from closely related species. The replication mechanism has usually evolved to optimize the extrachromosomal genome's success within the natural host and relies on the host for essential metabolites, enzymes, and other proteins as well as for the machinery for protein synthesis. Therefore, the fundamental tool of molecular cloning is always a two-component system—a compatible host-vector combination.

Recombination *in vitro* generally yields a population of DNA molecules, only some which have the desired structure. The cloning of unique constructs requires the following:

1. The conditions under which the population of recombinant DNAs is mixed with a population of recipient cells must favor the introduction of a single recombinant molecule into a recipient cell. This results in the separation of each recombinant from all the others.
2. Each recipient cell needs to be separated from all the others in the population to permit isolation of a clone of cells or viruses containing a unique recombinant.
3. Cells or viruses that receive recombinant DNAs must be distinguishable from those that do not so they can be selected for or identified by screening.
4. Cells that receive the desired recombinant must be distinguishable by screening or selection from those that contain other recombinant DNA molecules.

This chapter describes a variety of typical host-vector systems and deals with the first three requirements because the methods used for screening and selection largely depend on the particular properties of the host-vector combination being used. The techniques that allow a specific desired recombinant clone to be isolated—requirement 4—are described in Chapter 6. In general, we have selected examples that illustrate basic principles now being applied to the design of increasingly sophisticated systems for use in complex experimental situations.

The most widely used host-vector combinations involve *E. coli* strain K12 as host and *E. coli* plasmids and phage as vectors. *E. coli* K12 was an attractive choice because it had been the standard tool of microbial genetics long before the advent of recombinant DNA technology. Its